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Platelet-derived growth factor receptor signaling activates pericyte–myofibroblast transition in obstructive and post-ischemic kidney fibrosis

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Pericytes are the major source of scar-producing myofibroblasts following kidney injury; however, the mechanisms of this transition are unclear. To clarify this, we examined Collagen 1 (α1)-green fluorescent protein (GFP) reporter mice (pericytes and myofibroblasts express GFP) following ureteral obstruction or ischemia–reperfusion injury and focused on the role of platelet-derived growth factor (PDGF)-receptor (PDGFR) signaling in these two different injury models. Pericyte proliferation was noted after injury with reactivation of α-smooth muscle actin expression, a marker of the myofibroblast phenotype. PDGF expression increased in injured tubules, endothelium, and macrophages after injury, whereas PDGFR subunits α and β were expressed exclusively in interstitial GFP-labeled pericytes and myofibroblasts. When PDGFRα or PDGFRβ activation was inhibited by receptor-specific antibody following injury, proliferation and differentiation of pericytes decreased. The antibodies also blunted the injury-induced transcription of *PDGF*, *transforming growth factor β1*, and chemokine *CCL2*. They also reduced macrophage infiltration and fibrosis. Imatinib, a PDGFR tyrosine kinase inhibitor, attenuated pericyte proliferation and kidney fibrosis in both fibrogenic models. Thus, PDGFR signaling is involved in pericyte activation, proliferation, and differentiation into myofibroblasts during progressive kidney injury. Hence, pericytes may be a novel target to prevent kidney fibrosis by means of PDGFR signaling blockade.

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KEYWORDS: endothelium; fibroblast; pathophysiology of renal disease and progression; renal fibrosis; TGF-β

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We have recently identified microvascular pericytes as the primary source of collagen-producing myofibroblasts in progressively fibrotic kidneys following unilateral ureteral obstruction (UUO) or ischemia–reperfusion injury (IRI) using both kinetic mathematical modeling and state-of-the-art genetic fate-mapping studies.[1,2] Pericytes form direct communications with endothelial cells at peg and socket junctions where direct cell–cell interaction has been reported to occur.[3–5] With the increase of *collagen 1* (α1) (*Col1a1*) transcript, pericytes start to move away from capillaries, enter cell cycle, and subsequently increase in population size, within 24 h following kidney injury by UUO surgery.[1,6] All pericytes in the healthy adult kidney express platelet-derived growth factor receptor β (PDGFRβ) that continues to be expressed in all myofibroblasts in fibrotic kidney.[1,2,6] Although interrupting PDGFR signaling has been shown to result in significant delay in wound closure and attenuation of experimental kidney fibrosis,[7,8] it is unclear whether PDGFR signaling activates pericyte–myofibroblast transition in injured kidneys.

The fact that pericytes are a major source of myofibroblasts serves to readjust the focus of fibrosis research to areas that are potentially of more importance in the progression of kidney disease, including the cross-talk between pericytes and endothelial cells of the peritubular capillaries.[6,9] Indeed, ureteral obstruction of the kidney is known to result in downregulation of renal blood flow and glomerular filtration rate, suggesting that circulating factors may be important triggers activating pericyte responses.[10,11] Pericytes have been reported to serve as paracrine cells supporting vascular integrity and providing important angiogenic factors including vascular endothelial growth factor and angiopoietin 1.[12–14] Reciprocally, endothelial cells are the source of PDGF and transforming growth factor β1 (TGFβ1).[15] A lack of pericytes has been associated with aneurysm formation, leaky vessels, and spontaneous hemorrhage.[4,16] Vice versa, endothelial cell-derived PDGF is crucial for pericyte

recruitment along developing vasculature.[5,16] In addition to endothelial cells, tubular epithelial cells and recruited inflammatory macrophages are the important PDGF sources.[8,17]

The role of PDGFR signaling in kidney disease has been reviewed in detail.[8] PDGFR signaling consists of four ligands (PDGF-A, -B, -C, and -D) and two receptors (PDGFR-α and PDGFR-β). PDGFR possesses tyrosine kinase activity and is autophosphorylated upon ligand binding. Activated signaling will be transduced to activate various proto-oncogenes and immediate-early response genes responsible for particular functions.[18] The components of PDGF–PDGFR system are expressed constitutively or inducibly in kidneys, including endothelial cells, tubular epithelial cells, and interstitial cells.[19–25] Altered expression is characterized in almost all experimental and human kidney diseases. Intervention studies identified PDGF-C as a mediator of renal interstitial fibrosis and PDGF-B and PDGF-D as key factors involved in both mesangioproliferative disease and interstitial fibrosis.[23–26]

Collagen 1 (α1)-green fluorescent protein (GFP; *Coll-GFP*) reporter mice express high levels of GFP in interstitial pericytes and myofibroblasts in normal and fibrogenic kidneys, respectively. α-Smooth muscle actin (αSMA) is a robust marker of differentiated myofibroblasts in the adult kidney, but is not expressed by adult pericytes.[1,2] Using *Coll-GFP* mice, we aimed to study the expression of PDGFR subunits in pericytes and myofibroblasts, the role of PDGFR signaling in pericyte–myofibroblast transition in kidneys after ureteral obstruction or IRI, and whether blocking pericyte activation by PDGFR blockade may prevent interstitial kidney fibrosis.

RESULTS

PDGFRs are activated in kidneys after UUO surgery

PDGF isoform and *PDGFR* subunit transcript levels increased as UUO kidney injury progressed (Figure 1a), accompanied by marked increases in PDGFRα and PDGFRβ protein levels (Supplementary Figure S1 online). Western blot analyses for PDGF-B and PDGF-C, the two PDGF ligands implicated in interstitial fibrosis,[23,24] showed similar upregulation (Supplementary Figure S1A online). In *Coll-GFP* reporter mice, transcripts of *GFP* and *Col1a1* increased comparably in kidneys after UUO surgery (Supplementary Figure S2A online). Increased *Col1a1* transcript dictated the increase of Col1a1 protein in both Coll-GFP + myofibroblasts and extracellular area at day 4 after UUO surgery (Supplementary Figure S2B online). In normal *Coll-GFP* mice, GFP was detected in interstitial pericytes and glomerular podocytes of the kidney, whereas fibrogenic injury led to differentiation of pericytes into myofibroblasts (Figure 1b and c). Pericytes and interstitial myofibroblasts in normal and UUO kidneys, respectively, expressed PDGFRα and PDGFRβ exclusively, but did not express PDGF-B or PDGF-C (Figure 1b and d). Mesangium also expressed PDGFRβ, but PDGFRα was not detected in glomeruli (Figure 1c). PDGF-B isoform, the major ligand for receptors composed of PDGFRα and/or PDGFRβ, was not detected by immunofluorescence in normal kidney, whereas its expression was readily detected in tubules, endothelial cells, and macrophages of UUO kidney (Figure 1d and Supplementary Figure S3A and B online). PDGF-C isoform, the ligand for PDGFRα, was weakly detected in macrophages of normal kidney, and it had markedly increased expression in macrophages, but not in epithelial tubules or endothelial cells, of UUO kidney (Figure 1e and Supplementary Figure S3C online). Phosphorylation of PDGFRα and PDGFRβ increased with time after UUO surgery (Supplementary Figure S1 online). In normal kidney, a proportion of PDGFRβ is consistently phosphorylated, unlike PDGFRα.

Anti-PDGFR antibody inhibits activation of cognate PDGFR *in vitro* and *in vivo*

To verify the specificity of anti-PDGFRα (1E10) and anti-PDGFRβ (2C5) antibodies, we stimulated primary cultured UUO kidney myofibroblasts *in vitro* using PDGF-AA or PDGF-BB in the presence of anti-KLH control antibody (KLH), 1E10, or 2C5. Short-term stimulation increased receptor phosphorylation but not the level of total PDGFRα or PDGFRβ protein (Supplementary Figure S4 online). PDGF-AA-stimulated phosphorylation of both PDGFRα and PDGFRβ was inhibited by 1E10, indicating that 1E10 was specific for PDGFRα blockade and PDGF-AA-ligated PDGFRα might transactivate PDGFRβ (Supplementary Figure S4A online). PDGF-BB-stimulated phosphorylation of PDGFRβ was inhibited by 2C5 (Supplementary Figure S4B online). The expression of both PDGFRα and PDGFRβ increased in UUO kidneys of KLH-treated animals, whereas the increase in both receptors was attenuated in 1E10- or 2C5-treated mice. 1E10 and 2C5 in combination synergistically prevented PDGFRα and PDGFRβ phosphorylation (Figure 2a and b). Injury-stimulated phosphorylation of PDGFRα and PDGFRβ in UUO kidneys was also attenuated by either 1E10 or 2C5 (Figure 2a and c).

Anti-PDGFR antibody administration to mice attenuates kidney fibrogenesis

Because there is an exponential increase of interstitial Coll-GFP + cells from day 0 through day 4 and peak proliferation occurs at day 2 after UUO or IRI surgery,[1] we studied the inhibitory effect of anti-PDGFR antibodies on Coll-GFP + cells at day 4 and fibrosis at day 14 after surgery. Interstitial Coll-GFP + cells in UUO kidneys of mice administered with KLH control antibody increased in population markedly, and 97.4% of them expressed α-SMA by immunofluorescence, indicative of myofibroblast differentiation at day 4 after surgery (Figure 3a–c). Administration of 1E10 and 2C5 antibodies decreased the number of interstitial Coll-GFP + cells by 36 and 43%, respectively (Figure 3a and b). This inhibition was recapitulated in the cell number of αSMA + ;Coll-GFP + myofibroblasts (Figure 3a and c). Compared with 1E10 or 2C5 treatment alone, no

enhanced effect of combining both anti-PDGFR antibodies on the myofibroblast population was noted (Figure 3b and c). Only 0.9% of total kidney cells were Coll-GFP + ;PDGFRα + in normal kidney analyzed by flow cytometry, whereas the percentage increased markedly to 12.4% in UUO kidney of KLH-treated mice (Supplementary Figure S5 online). Treatment with 1E10 or 2C5 antibodies decreased the percentage to 7.3 and 7.7%, respectively (Supplementary

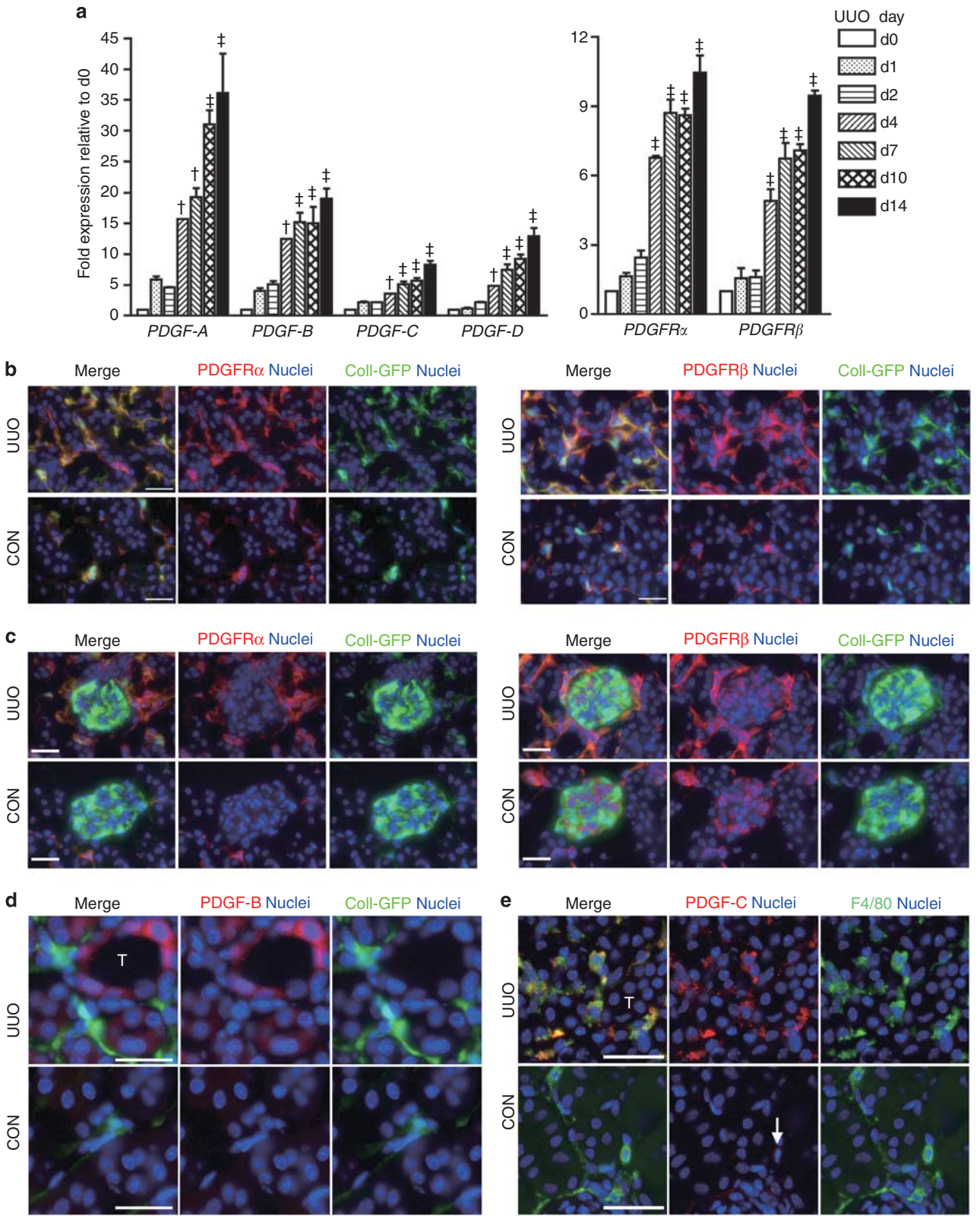

Figure S5 online). To determine myofibroblast differentiation, Coll-GFP+;PDGFRα+ cells were further analyzed for αSMA expression by flow cytometry. Very low level of αSMA was detected in 0.7% of Coll-GFP+;PDGFRα+ cells in normal kidney, whereas the expression was increased and detected in 71.2% of Coll-GFP+;PDGFRα+ cells in UUO kidney of KLH-treated mice (Figure 3d and e). Antibodies 1E10 and 2C5 decreased not only the αSMA-expressing cells to 47.4 and 44.4%, respectively, but also the mean peak fluorescence level (Figure 3d and e). Quantitative PCR (Q-PCR) showed a 15- to 20-fold increase of *Col1a1*, *Col3a1*, and *αSMA* transcripts in UUO kidney compared with normal kidney, and this was markedly downregulated by either 1E10 or 2C5 administration (Figure 4a). 1E10 together with 2C5 produced no significant additional effect. Expression of whole kidney αSMA protein or GFP protein was attenuated by 1E10 or 2C5 antibodies (Figure 4b). To study the effect of anti-PDGFR antibody on differentiation of pericytes into myofibroblasts, we purified pericytes or myofibroblasts by sorting Coll-GFP+;PDGFRα+ cells from normal or UUO kidneys, respectively, in antibody-treated animals (Supplementary Figure S5A online). Q-PCR showed 3- and 18-fold increase of *Col1a1* and *αSMA* transcripts, respectively, in UUO kidney myofibroblasts purified from KLH-treated animals, which were markedly downregulated in myofibroblasts purified from either 1E10- or 2C5-treated animals (Figure 4c). Pericytes in normal kidney did not express detectable αSMA protein by immunofluorescence, although very low levels of *αSMA* transcript were detected by Q-PCR (Figures 3a and c and 4c). The consequence of inhibition of pericyte–myofibroblast differentiation and *Col1a1* transcription in myofibroblasts was a 70% attenuation of kidney fibrosis at 14 days after UUO surgery with 1E10 or 2C5 antibodies compared with KLH-treated animals (Supplementary Figure S6 and Figure 4d online). Both 1E10 and 2C5 antibodies decreased the collagen deposition significantly (Supplementary Figure S6 and Figure 4d online). Next, we detected the pan cell-cycle marker Ki67. In KLH-treated mice, 32.2% of interstitial Coll-GFP+ cells in UUO kidneys were in cell cycle day 4 after surgery (Supplementary Figure S7 and Figure 5a online). Administration of 1E10 or 2C5 antibodies decreased the number of proliferating interstitial Coll-GFP+ cells by 34 and 38%, respectively (Supplementary Figure S7 and Figure 5a online). Both PDGFR antibodies inhibited upregulation of *PDGF* isoform and *TGFβ1* transcripts in UUO kidneys (Figure 5b–f).

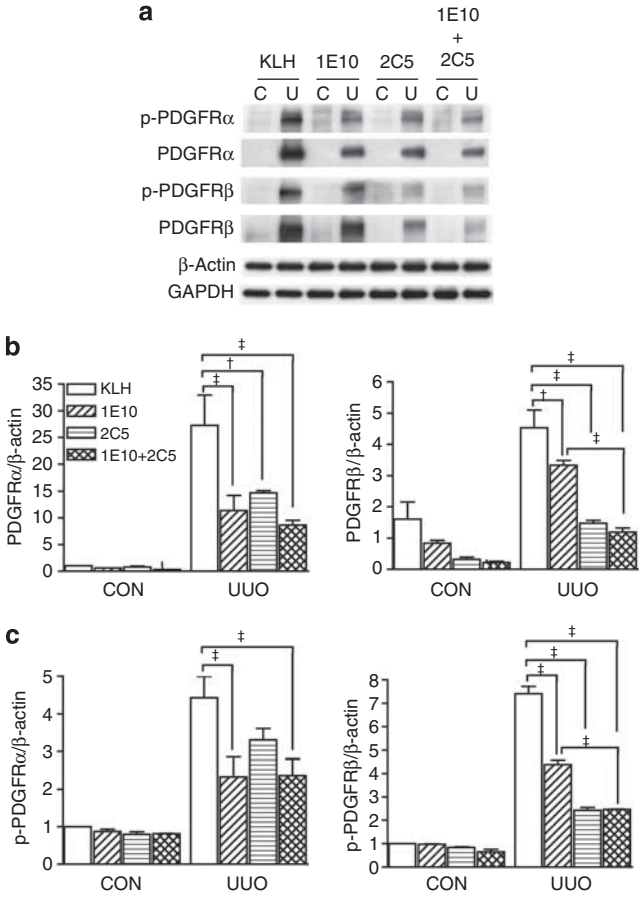

Figure 2 | Anti-platelet-derived growth factor receptor (PDGFR) antibody inhibited PDGFR expression. (**a**) Representative images were that of western blot of whole kidney lysates for tyrosine phosphorylation of PDGFRα (p-PDGFRα) and PDGFRβ (p-PDGFRβ), and their total forms from mice treated with control (KLH), anti-PDGFRα (1E10), anti-PDGFRβ (2C5), or combination treatment (1E10 + 2C5). C and U denote control and UUO kidneys at day 4 after surgery, respectively. (**b**, **c**) Expression levels were normalized by β-actin. $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$. $N=6$/group.

TGFβ1, not PDGF, stimulates myofibroblast differentiation from pericytes *in vitro*

UUO kidney myofibroblasts cultured *in vitro* on glass appeared spindle shaped and expressed αSMA and vimentin in the absence or presence of PDGF or TGFβ1 (Figure 6a). However, only TGFβ1 further increased *Col1a1* and *αSMA* transcripts (Figure 6b). Pericytes purified from normal

Figure 1 | Platelet-derived growth factor (PDGF) and PDGF receptor (PDGFR) increased expression in kidneys after unilateral ureteral obstruction (UUO). (**a**) Quantitative PCR (Q-PCR) time course for transcripts of whole kidney *PDGF isoforms* and *PDGFR subunits* following UUO surgery. Expression levels were normalized by *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*). $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$ versus normal kidney at day 0 (d0). $N=3$/time point. (**b**) Immunofluorescence images showing that PDGFRα and PDGFRβ were expressed exclusively in interstitial Coll-GFP+ cells of normal control (CON) and UUO kidneys at day 4 after surgery. (**c**) Immunofluorescence images showing that PDGFRβ, but not PDGFRα, was expressed in glomerular mesangium of CON and UUO kidneys at day 4 after surgery in *Coll-GFP* mice whose glomerular podocytes expressed Coll-GFP. (**d**) Immunofluorescence images showing PDGF-B in epithelial cells of dilated tubules of day 4 UUO kidney (denoted by T), not in Coll-GFP+ cells. PDGF-B was difficult to detect in CON kidney. (**e**) Immunofluorescence images showing that PDGF-C was expressed in F4/80+ macrophages of day 4 UUO kidney, but in a very low level in F4/80+ macrophages of CON kidney (arrow) and not in tubular epithelial cells (denoted by T). Scale bar = 25 μm.

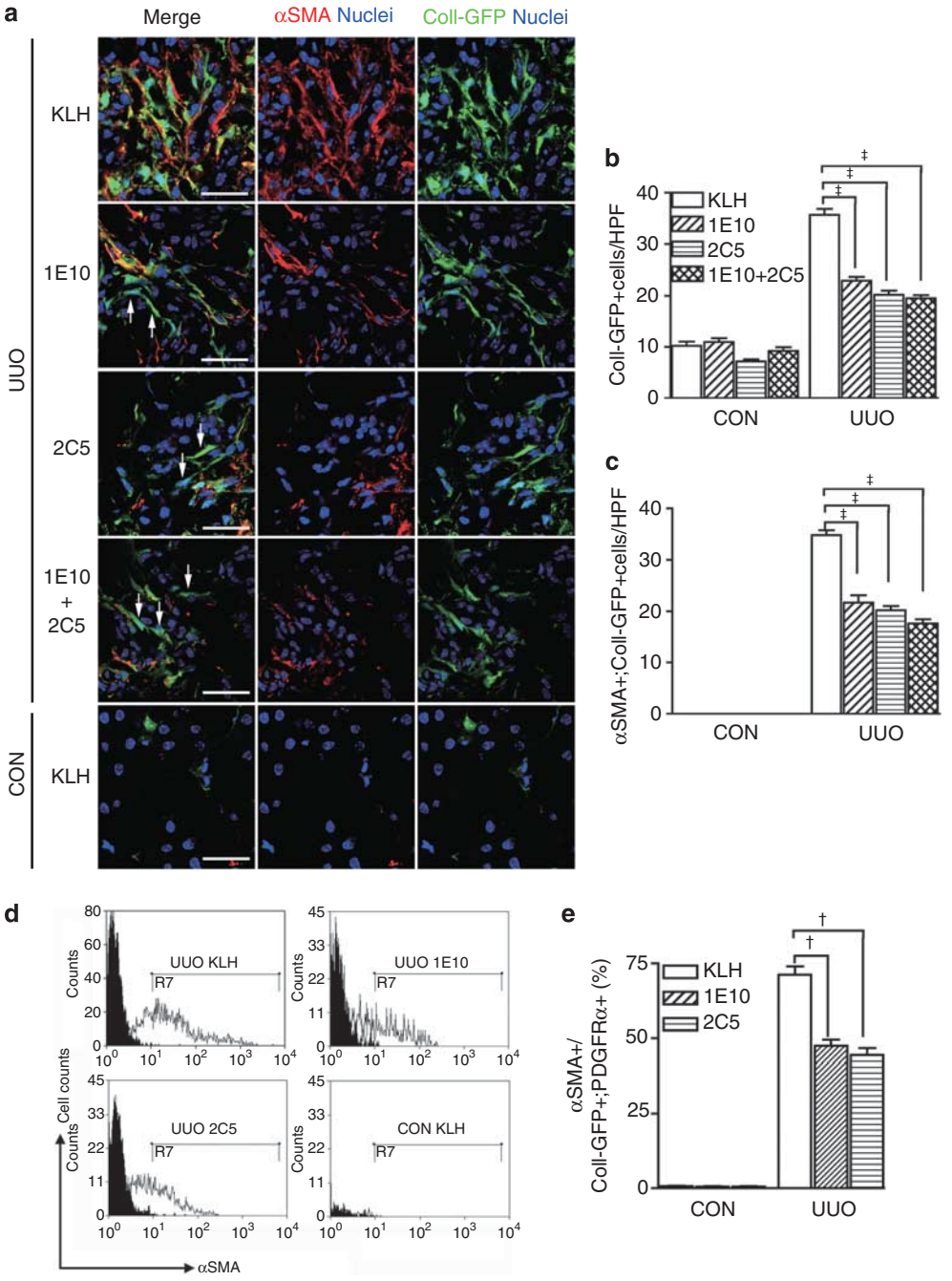

Figure 3 | Anti-platelet-derived growth factor receptor (PDGFR) antibody inhibited pericyte–myofibroblast transition. (**a**) Confocal microscopic images of interstitial Coll-GFP + cells colabeled with myofibroblast marker α-smooth muscle actin (αSMA) in kidneys at day 4 after surgery. Arrows indicate αSMA−;Coll-GFP + cells. Scale bar = 25 μm. (**b**, **c**) Quantification of (**b**) interstitial Coll-GFP + cells and (**c**) αSMA + ;Coll-GFP + myofibroblasts in high-powered field (HPF) at × 400 magnification. $N = 6$/group. (**d**, **e**) Fluorescence-activated cell sorting (FACS) analysis showing αSMA expression (white) in Coll-GFP + ;PDGFRα + cells of control (CON) and unilateral ureteral obstruction (UUO) kidneys from mice treated with KLH, 1E10, or 2C5. Coll-GFP + ;PDGFRα + cells were sorted as described in Supplementary Figure S5A online. Isotype control is shown in black. (**d**) Representative plots are one of three independent experiments. Quantification of αSMA + cells in Coll-GFP + ;PDGFRα + cells (%) analyzed by FACS is shown in **e**. $N = 3$/group. $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$.

kidneys of *Coll-GFP* mice and cultured on glass were spindle shaped and continued to express GFP (Figure 6c). All pericytes expressed vimentin, but αSMA was not detected. After stimulation by TGFβ1, but not by PDGF, αSMA was markedly increased at both the protein and transcriptional levels (Figure 6c and d).

Anti-PDGFR antibody decreases macrophage infiltration

A fivefold increase of F4/80 + macrophages was noted in UUO kidneys of KLH-treated mice at day 4 after surgery (Figure 7a and b). Administration of 1E10 or 2C5 antibodies resulted in reduction of macrophage cell number by 60 and 54%, respectively (Figure 7a and b). Q-PCR for *CCR2*

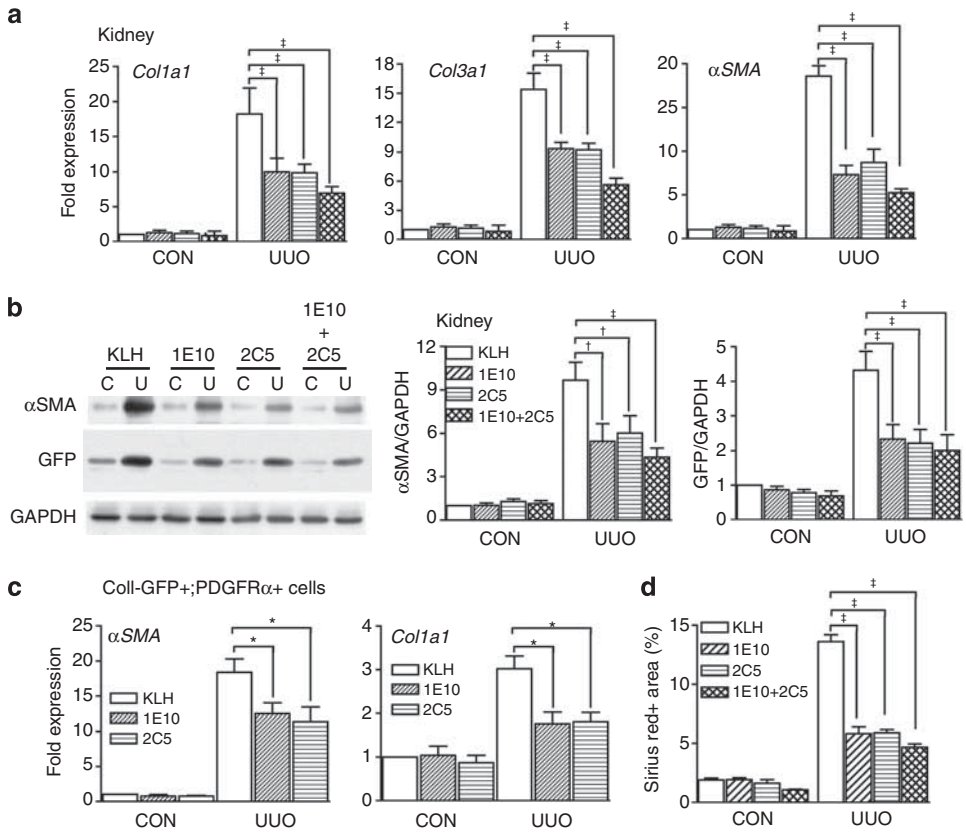

Figure 4 | Anti-platelet-derived growth factor receptor (PDGFR) antibody attenuated kidney fibrosis. (**a**) Quantitative PCR (Q-PCR) of *collagen 1* (α1) (*Col1a1*), *collagen 3* (α1) (*Col3a1*), and α-*smooth muscle actin* (α*SMA*) transcripts of control (CON) and day 4 unilateral ureteral obstruction (UUO) kidneys in mice treated with antibody as indicated. $N = 6$/group. (**b**) Representative images were that of western blot of whole kidney lysates for αSMA, GFP, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). C and U denote control and UUO kidneys, respectively. Expression levels were normalized by GAPDH. $N = 6$/group. (**c**) Q-PCR of α*SMA* and *Col1a1* transcripts of purified Coll-GFP + ;PDGFRα + cells from CON and day 4 UUO kidneys in mice treated with antibody as indicated. $N = 4$/group. (**d**) Morphometric quantification of fibrillar collagen from whole sagittal kidney section in mice treated with antibody as indicated. $^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$.

(*chemokine* (*C-C motif*) *receptor 2*) and *CX3CR1* (*CX3C chemokine receptor 1*) transcripts in UUO kidneys indicated that upregulation of these myeloid-restricted transcripts in KLH-treated mice was attenuated by either 1E10 or 2C5 antibodies (Figure 7c and d). In addition, either of the anti-PDGFR antibodies inhibited the injury-stimulated upregulation of monocyte–macrophage chemoattractant *CCL2* (*chemokine* (*C-C motif*) *ligand 2*) transcripts (Figure 7e). We were intrigued by the possibility that macrophages possess receptors that enable a direct response to PDGF ligands. Unlike kidney myofibroblasts, bone marrow-derived macrophages, when analyzed by flow cytometry, did not express PDGFRα or PDGFRβ (Supplementary Figure S8A online), and confocal microscopic images of immunostained sections did not detect PDGFRα or PDGFRβ in kidney macrophages *in vivo* (Supplementary Figure S8B online).

Imatinib inhibits PDGFR signaling in pericytes and attenuates kidney fibrosis

After demonstrating that disruption of PDGFR signaling in pericytes attenuated kidney fibrosis by receptor-specific antibodies, we tested the efficacy of a clinically applicable tyrosine kinase inhibitor, imatinib mesylate, in the same kidney fibrosis model. Compared with vehicle, imatinib decreased both PDGFRα and PDGFRβ phosphorylation and Coll-GFP expression in UUO kidneys (Figure 8a and b). Imatinib decreased the expanded population of both Coll-GFP + cells and αSMA + ;Coll-GFP + myofibroblasts by inhibiting cell proliferation (Figure 8c–e and Supplementary Figures S9 and S10 online), and attenuated macrophage infiltration (Figure 8f and Supplementary Figure S11 online) and fibrosis development in UUO kidneys (Figure 8g and Supplementary Figure S12 online). These antifibrogenic properties could not be attributed to c-kit inhibition in pericytes–myofibroblasts because these cells do not express c-kit (Supplementary Figure S13 online).

Blockade of PDGFR signaling attenuates IRI-induced kidney fibrogenesis

To determine whether the antifibrogenic properties of PDGFR signaling blockade is generalizable to other models of cortical and medullary injury to the kidney, we studied their properties in a second fibrogenic model that we have

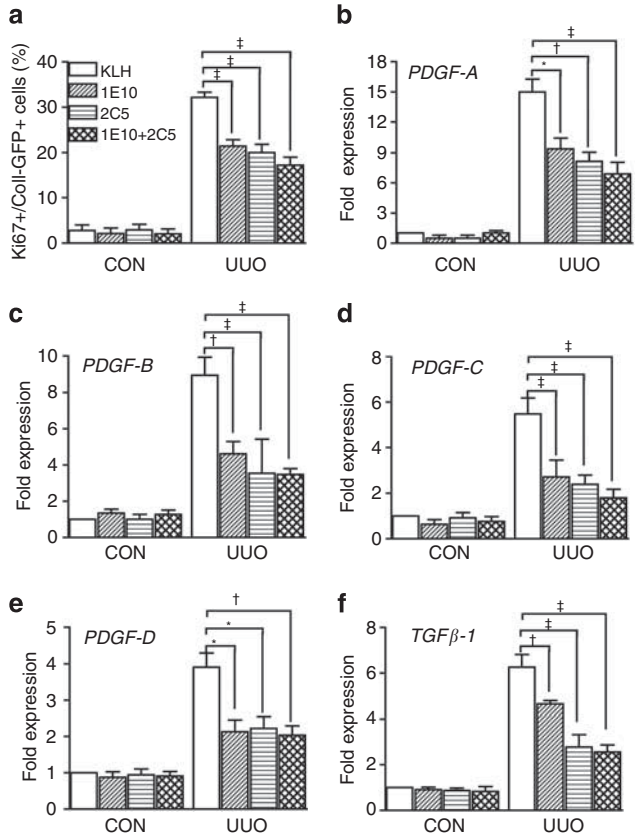

Figure 5 | Anti-platelet-derived growth factor receptor (PDGFR) antibody inhibited injury-stimulated proliferation of pericytes–fibroblasts and gene expression of profibrotic growth factors. (**a**) Quantification of the percentage of Coll-GFP + cells with positive Ki67 costaining. $N = 6$/group. $^{\ddagger}P < 0.001$. (**b–f**) Quantitative PCR (Q-PCR) of *PDGF isoforms* and *transforming growth factor β1* (*TGFβ1*) transcripts in kidneys at day 4 after unilateral ureteral obstruction (UUO) surgery. $N = 6$/group. $^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$.

previously reported, the unilateral IRI model.[1,6,27] Single kidneys exposed to IRI develop progressive interstitial fibrosis by day 14. Similar to the studies on UUO injury, IRI kidneys from mice with imatinib administration experienced considerably fewer total interstitial Coll-GFP + cells and fewer αSMA + ;Coll-GFP + myofibroblasts on days 4 and 14 post IRI (Supplementary Figure S14A–D online). The consequence of blocking of Coll-GFP + myofibroblast appearance was marked attenuation of interstitial fibrosis on day 14 post IRI (Supplementary Figure S14E and F online), similar to our observations in the UUO kidney injury model.

DISCUSSION

These studies highlight the efficacy of strategies to block PDGFRα and PDGFRβ signaling in response to ischemic or mechanical injury in mouse adult kidney. Antibody-mediated blockade or small-molecule inhibition of tyrosine phosphorylation of the PDGFRs is sufficient to attenuate the development of interstitial fibrosis by >70%. Moreover, single receptor blockade prevents recruitment of inflammatory macrophages to the kidney. Macrophages are inflammatory cells that contribute to progression of fibrosis in these models.[17,27] These studies therefore point to PDGFRα or PDGFRβ as potential candidates for therapies in human ischemic or other kidney injuries.

In our experiments, PDGFRα is exclusively expressed by kidney pericytes and PDGFRβ is expressed by kidney pericytes, as well as by mesangial cells of the glomerulus and vascular smooth muscle of kidney arterioles. Strikingly, these receptors are neither expressed by recruited leukocytes nor by epithelium or endothelium. Therefore, it is notable in the injury models reported here, which do not focus on the glomerulus, that targeting single receptors restricted to pericytes is sufficient to prevent fibrosis and inflammation. These findings support previous studies that have identified pericytes of the peritubular capillaries as the major source of myofibroblasts.[1,2]

In accordance with previous studies,[19–25] PDGF ligand expression was low in normal kidneys, but increased markedly and rapidly in endothelial cells, tubular epithelial cells, and macrophages after ureteral ligation. Although 1E10 and 2C5 antibodies specifically inhibited PDGF-stimulated activation of PDGFRα and PDGFRβ *in vitro*, respectively, each produced comparable inhibition of phosphorylation of both PDGFRα and PDGFRβ *in vivo*. This might be the consequence of their comparable capacity to inhibit the expression of *PDGF* isoforms induced by injury, especially *PDGF-B* and *PDGF-C*, the two important PDGF ligands previously implicated in interstitial fibrosis. Therefore, either anti-PDGFRα or anti-PDGFRβ antibody administration has the capacity to cross-inhibit the other receptor-signaling pathway *in vivo* through downregulation of PDGF ligands.

Microvascular Coll-GFP + pericytes did not express αSMA in normal kidney, whereas, following ureteral obstruction, almost all interstitial Coll-GFP + cells expressed αSMA indicative of differentiation to myofibroblasts at 4 days after injury onset. These studies are the first to show that anti-PDGFR antibody administration markedly decreased the number of αSMA + myofibroblasts in UUO kidneys, and in addition show, by flow cytometry, that anti-PDGFR antibody treatment also downregulates overall αSMA expression in individual purified Coll-GFP + ;PDGFRα + cells from UUO kidney. *In vivo*, there was a role of PDGFR signaling in inducing αSMA expression and myofibroblast differentiation. *In vitro* cultured pericytes increased αSMA expression after TGFβ1 stimulation but not after PDGF treatment, suggesting that PDGF alone might not be sufficient to induce pericyte–myofibroblast transition. Our data showed that anti-PDGFR antibody treatment inhibited upregulation of TGFβ1 in injured kidney. This finding supports a hypothesis that TGFβ1 may be an important contributing cytokine in transducing activated PDGFR signaling in pericyte–myofibroblast transition *in vivo*.[28,29] Further studies will be required to dissect this potential

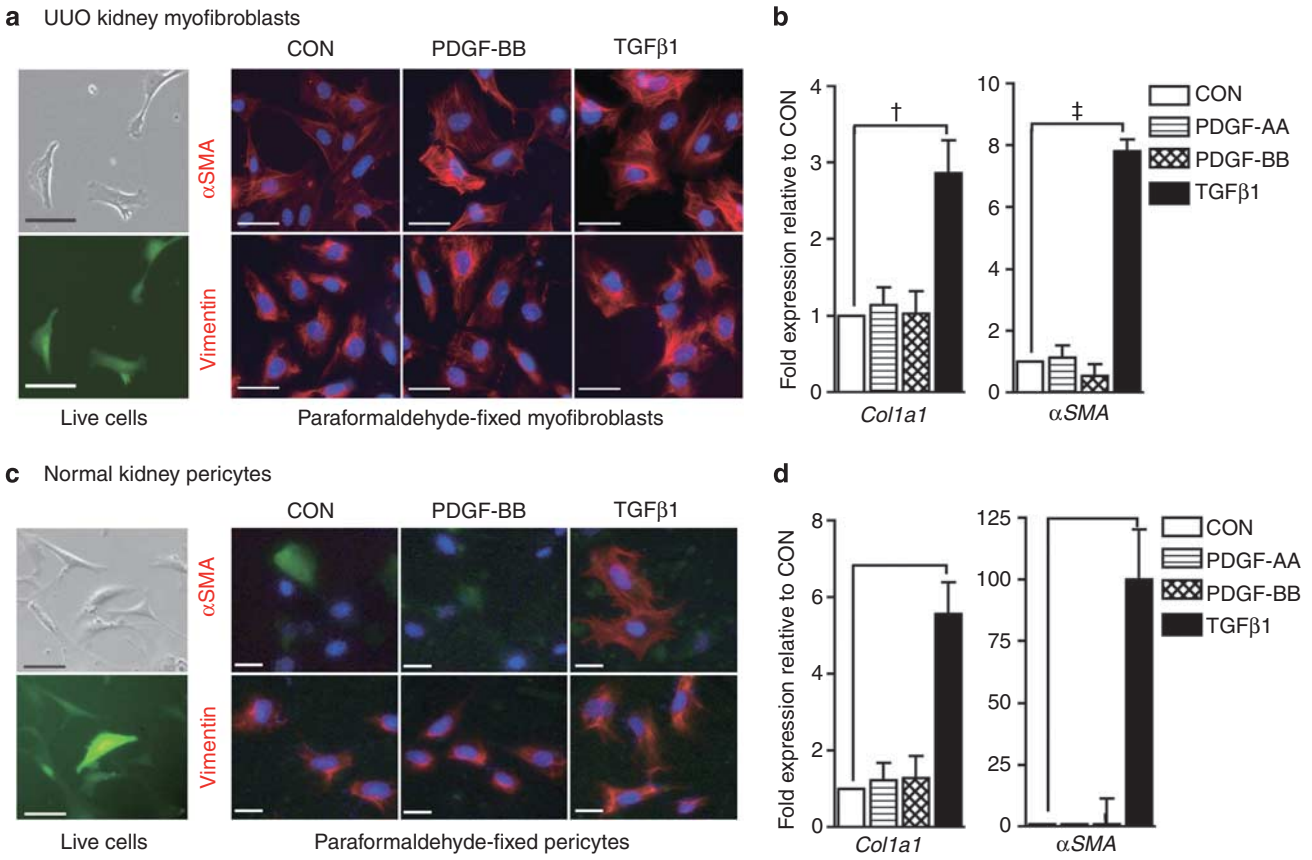

Figure 6 | Transforming growth factor β1 (TGFβ1), not platelet-derived growth factor (PDGF), induced pericyte–myofibroblast transition *in vitro*. (**a**) Bright-field and fluorescence images showing cultured live unilateral ureteral obstruction (UUO) kidney myofibroblasts at passage 6. Immunofluorescence images showing α-smooth muscle actin (αSMA) and vimentin staining in fixed myofibroblasts with different treatments as indicated. Representative images are one of three independent experiments. Scale bar = 25 μm. (**b**) Quantitative PCR (Q-PCR) of *collagen 1* (α*1*) (*Col1a1*) and α*SMA* transcripts of cultured UUO kidney myofibroblasts with different treatments as indicated. $N = 3$/group. (**c**) Bright-field and fluorescence images showing primary cultured live normal kidney pericytes at passage 0. Immunofluorescence images showing αSMA and vimentin staining in fixed pericytes with different treatments as indicated. Scale bar = 25 μm. (**d**) Q-PCR of *Col1a*α*1* and α*SMA* transcripts of primary cultured pericytes with different treatments as indicated. $N = 3$/group. $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$.

cooperation of these cytokine-signaling pathways in the pericyte.

It is increasingly clear that renal fibrosis is not only a passive deposition of extracellular matrix but also involves active inflammation.[30] Consistent with this, a significant fibrosis reduction has been demonstrated in murine renal fibrosis models through selective macrophage ablation or functional regulation, by chemokine mutation or blockade of chemokine receptors.[17,27,31–33] PDGFR signaling activated by PDGF-B or PDGF-C has been shown to regulate the production of proinflammatory chemokines by renal myofibroblasts including CCL2 and CCL5.[24,34] In agreement with this, our data showed that injury-stimulated expression of *CCL2* was inhibited by PDGFR antibody. Both *CCR2* and *CX3CR1* transcripts were downregulated by PDGFR blockade, suggestive of a reduction of both proinjurious and profibrotic macrophage subpopulations.[17,27] Our studies here indicate that PDGF ligands are not mitogens for macrophages, because macrophages expressed neither PDGFRα nor PDGFRβ. Therefore, the inhibition of macrophage infiltration was most likely secondary to the downregulation of chemokines. Our previous studies have shown that macrophages, especially $Ly6C^{low}$ macrophages (also known as the $CX3CR1^{high}$ sub-population), could stimulate myofibroblast proliferation and extracellular matrix production in progressive kidney or liver fibrosis.[17,27,31,35] $Ly6C^{low}$ macrophages are one of the major cell types in the interstitium producing both PDGF ligands and TGFβ1.[17] In these studies, the decrease in inflammatory macrophages, triggered by PDGFR-specific antibodies, is sufficient to account for the downregulation of injury-stimulated PDGF and TGFβ1.

Although the small-molecule imatinib was developed primarily to inhibit c-abl kinase, it also inhibits PDGFR kinase activity, and therefore signaling, at comparable half-maximal inhibitory concentration.[36] Its beneficial effect has been demonstrated in many animal models of kidney disease, including UUO kidney fibrosis in rats,[37] lupus nephritis, cryoglobulinemic glomerulonephritis, and diabetic nephropathy in mice.[38–40] In addition to reducing macrophage infiltration, this study identified that imatinib attenuates

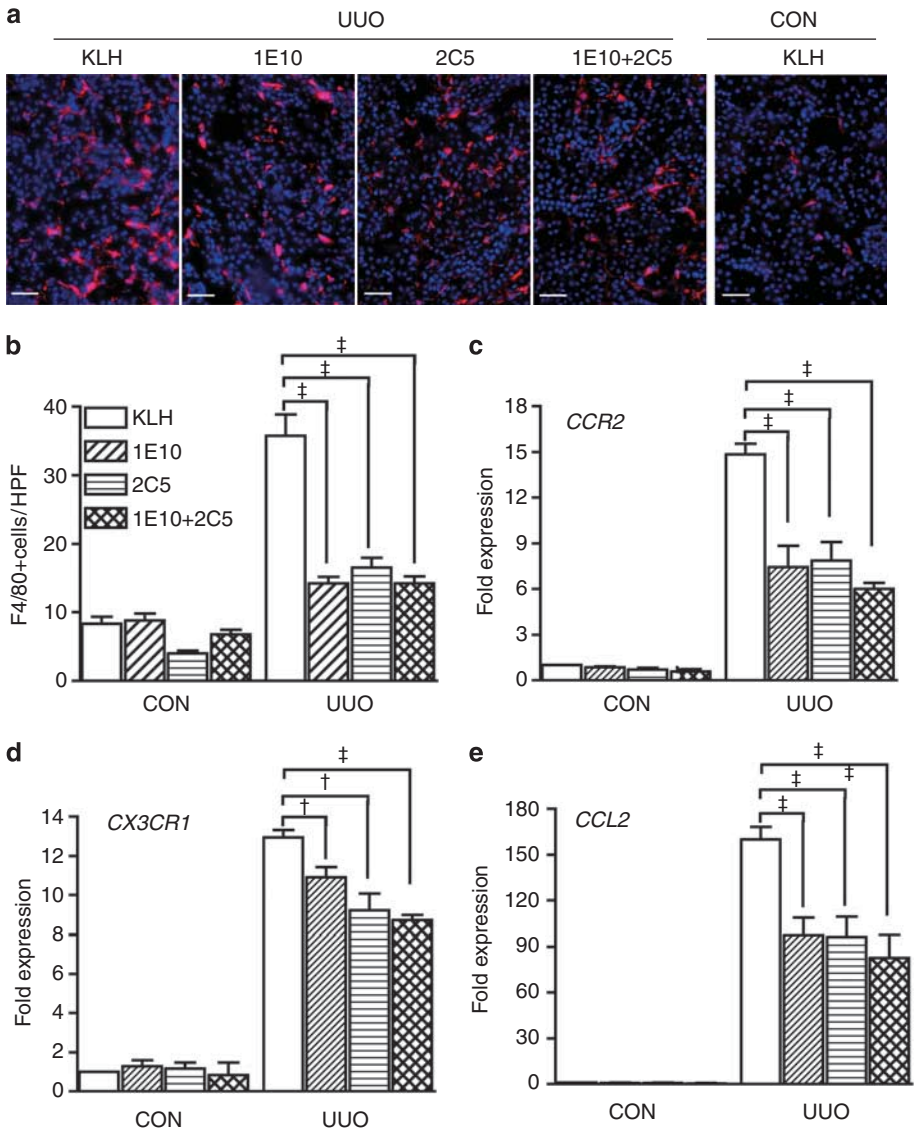

Figure 7 | Anti-platelet-derived growth factor receptor (PDGFR) antibody inhibited macrophage infiltration. (**a**) Immunofluorescence micrographs showing interstitial F4/80+ macrophages (red) in kidneys at day 4 after surgery. Scale bar = 25 μm. (**b**) Quantification of F4/80+ macrophages. (**c**–**e**) Quantitative PCR (Q-PCR) of monocyte–macrophage chemokine transcript *CCL2* (*chemokine* (*C-C motif*) *ligand 2,* also known as *monocyte chemoattractant protein-1* (*MCP1*)), and chemokine receptor transcripts *CCR2* (*chemokine* (*C-C motif*) *receptor 2*) and *CX3CR1* (*CX3C chemokine receptor 1*) in kidneys at day 4 after surgery. $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$. $N=6$/group.

kidney fibrosis by inhibiting pericyte activation in injured kidneys. Our findings suggest strongly that this beneficial effect on the kidney is due to the direct inhibition of PDGFR tyrosine kinase activity in pericytes, as pericytes were not found to express the receptor c-kit. However, c-kit was identified in epithelial cells reproducing findings reported elsewhere.[41] It is currently not clear whether c-kit inhibition on tubular epithelial cells by imatinib has any bearing on its antifibrogenic properties.

Although reports have previously identified epithelial cells and circulating cells as major direct contributors to kidney myofibroblasts,[42,43] extensive studies from our laboratories, those of our collaborators, and several independent investigators, using different state-of-the-art genetic fate-mapping strategies and other stringent techniques, in several different models of kidney injury, have not supported those earlier reports.[1,2,6,9,27,44–56] Our laboratories and those of our collaborators only find kidney pericytes and perivascular fibroblasts derived from embryonic FoxD1+ cap metanephric mesenchyme as cells that become scar-forming myofibroblasts. This Discussion is not the appropriate forum to review and debate the newly published fate-mapping findings with the earlier studies, as these have already been discussed in many peer-reviewed journals.[1,2,6,9,27,44–56] Regardless of the precise origins of myofibroblasts, the studies presented in these pages, inspired by the newly discovered pathological role of kidney pericytes, firmly and unequivocally identify a large population of PDGFRα- and PDGFRβ-expressing cells that are kidney pericytes, and blockade of the receptors on these cells prevents development of interstitial inflammation

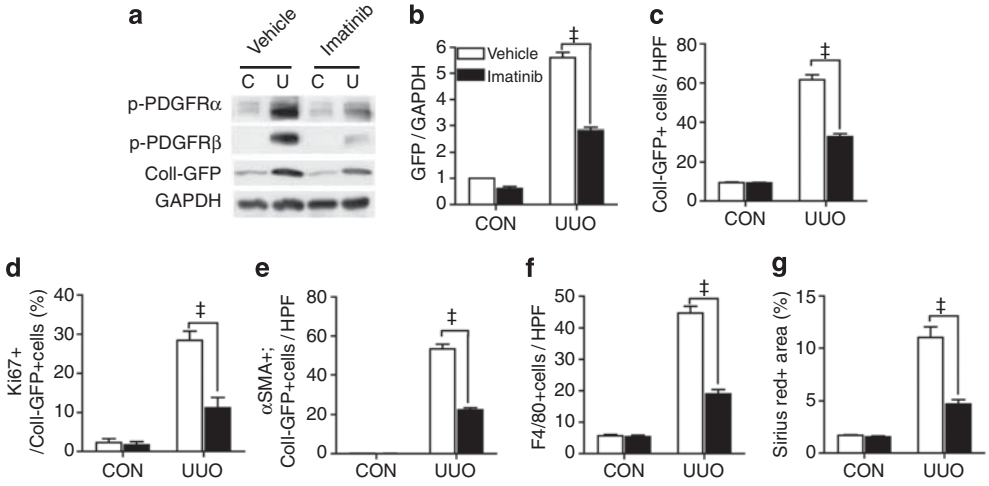

Figure 8 | Imatinib inhibited pericyte platelet-derived growth factor receptor (PDGFR) signaling and attenuated kidney fibrosis. (**a**, **b**) The *in vivo* inhibitory effect of imatinib on PDGFR activation and Coll-GFP expression is shown by western blot analyses (**a**). Coll-GFP normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in unilateral ureteral obstruction (UUO) kidneys was downregulated by imatinib (**b**). (**c**–**f**) Imatinib inhibited the (**c**) expanded population and (**d**) cell proliferation of the interstitial Coll-GFP+ cells, (**e**) expanded population of the interstitial αSMA+;Coll-GFP+ myofibroblasts, as well as (**f**) F4/80+ macrophages in UUO kidneys at day 4 after surgery. (**g**) Imatinib attenuated picrosirius red-stained fibrillar collagens in kidneys at day 14 after UUO surgery. ‡$P<0.001$ ($N=6$/group).

and kidney fibrosis in two clinically relevant models of kidney injury in mice.

In conclusion, this report provides new evidence that activated PDGFR signaling leads to the proliferation and differentiation of pericytes into myofibroblasts in progressive kidney disease and identifies pericytes as a novel therapeutic target to prevent fibrosis development by PDGFR blockade.

MATERIALS AND METHODS

Mouse models of kidney fibrosis

UUO was performed in adult (8–12 weeks) C57BL6 wild-type or *Coll-GFP* mice as described previously.[1] Briefly, the left ureter was ligated twice using 4-0 nylon surgical sutures at the level of the lower pole of the kidney. The unilateral IRI model was performed as previously described using a 30-min ischemic time at 36.8–37.3 °C core temperature.[27] All animal studies were carried out under a protocol approved by the institutional animal care and use committee, National Taiwan University, College of Medicine.

Coll-GFP mice

Coll-GFP transgenic mice generated on the C57BL6 background were as described previously.[1] In brief, 3.2 kb of the *Col1a1* promoter and enhancer with the open reading frame of EGFP yielded highest levels of GFP expression when *Col1a1* transcripts were generated.

Anti-PDGFR antibody treatment

Mice were treated with control (KLH), anti-PDGFRα (1E10), anti-PDGFRβ (2C5), or a combination of 1E10 and 2C5 (40 mg/kg intraperitoneally, ImClone Systems, New York, NY) 2 h before surgery, and then every other day until killing on days 4 and 14 ($N=6$ for each group).[57,58]

Imatinib mesylate treatment

Mice were treated by gavages with vehicle or imatinib mesylate (50 mg/kg, Novartis Pharmaceuticals, Basel, Switzerland) 2 h before surgery, and then twice a day until killing on days 4 and 14 ($N=6$ for each group).

Purification and culture of pericytes from normal kidney

Pericyte purification from normal kidney was described previously.[1] Briefly, the kidney was diced, incubated at 37 °C for 30 min with liberase (0.5 mg/ml, Roche, Mannheim, Germany) and DNase (100 U/ml, Roche) in Hank's buffered salt solution. After centrifugation, cells were resuspended in 5 ml of phosphate-buffered saline/1% bovine serum albumin, and filtered (40 μm). Pericytes were purified by isolating GFP+;PDGFRα+ cells using FACSAria cell sorting (BD Biosciences, San Jose, CA), and then total RNA was isolated or cultured in Dulbecco's modied Eagle's medium with 20% fetal bovine serum. After 4 days, the pericytes were then treated with PDGF-AA, PDGF-BB, or TGFβ1 (10 ng/ml, R&D Systems, Minneapolis, MN), or no additional treatment in Dulbecco's modied Eagle's medium with 1% fetal bovine serum. After 2 days, cells were fixed with 4% paraformaldehyde for 5 min for immunofluorescence study or subjected to total RNA extraction.

Purification and culture of myofibroblasts from kidney

Purification of kidney myofibroblasts from kidney at day 7 after UUO was described previously.[1] The kidney was diced and incubated at 37 °C for 1 h with liberase (0.5 mg/ml) and DNase (100 U/ml) in Hank's buffered salt solution. After centrifugation, cells were resuspended in 5 ml of phosphate-buffered saline/1% bovine serum albumin and filtered (40 μm). Myofibroblasts were purified by isolating GFP+;PDGFRα+ cells using FACSAria cell sorting and then total RNA was isolated, or purified cells were cultured in Dulbecco's modied Eagle's medium with 20% fetal bovine serum. The primary cultured cells used in this study were between passages 4 and 6 and have been characterized previously.[1]

Fluorescence-activated cell sorting (FACS) analysis

Bone marrow-derived macrophages and UUO kidney myofibroblasts were resuspended in FACS buffer and incubated with antibodies against PDGFRα, PDGFRβ, CD11b, or F4/80

(1:200, eBioscience, San Diego, CA) for 30 min at 4 °C in the presence of 1% mouse serum. Cells were then analyzed using BD FACSCalibur flow cytometer.[17] To analyze αSMA expression in kidney Coll-GFP+;PDGFRα+ cells, single cells were fixed in 4% paraformaldehyde/phosphate-buffered saline for 10 min with shaking at 4 °C, and then permeabilized in 0.1% saponin/phosphate-buffered saline for 10 min. After washing, the single cells were incubated with antibodies against PDGFRα (1:200, eBioscience), αSMA, or isotype control (1:20, R&D Systems) for 30 min. Thereafter, cells were analyzed using flow cytometer.

In vitro analysis of anti-PDGFR antibody

UUO kidney myofibroblasts were cultured in Dulbecco's modied Eagle's medium with 0.5% fetal bovine serum for 16 h, and then incubated with PDGF-AA or PDGF-BB (10 ng/ml, R&D Systems) in the presence of KLH, 1E10, or 2C5 (100 μg/ml) for 15 min. The extent of PDGFRα or β phosphorylation was determined by western blot analysis.

Tissue preparation and histology

Mouse tissues were prepared and stained as previously described.[1] Primary antibodies against the following proteins were used for immunolabeling: F4/80, CD31 (1:200, eBioscience), Ki-67, PDGF-B (1:200, Abcam, Cambridge, UK), vimentin, PDGF-C (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), and αSMA-Cy3 (1:200, clone 1A4, Sigma, St Louis, MO). Fluorescent-conjugated secondary antibody labeling (1:400–1:800, Jackson Immunoresearch, West Grove, PA), colabeled with DAPI (4,6-diamidino-2-phenylindole), mounting with Vectashield/DAPI (Vector Laboratories, Burlingame, CA), image capture, and processing were carried out as previously described. Quantification of specific cells in tissue sections was carried out as described previously.[6] Specific cells were counted in 10 cortical interstitial fields randomly selected at ×400 magnification (high-powered field) per mouse. Interstitial fibrosis was quantified in picrosirius red-stained paraffin sections.

Quantitative-PCR

cDNA was synthesized using oligo(dT) and random primers. Conventional PCR and Q-PCR were performed using methods described previously.[1] The specific primer pairs used in PCR are listed in Supplementary Table S1 online.

Western blot analysis

Total cellular protein was subjected to western blot analysis using methods described previously.[59] The following primary antibodies were used to detect the specific protein: Tyr754 phospho-PDGFRα, Tyr751 phospho-PDGFRβ, PDGFRα and PDGFRβ (Cell Signaling Technology, Beverly, MA), PDGF-B (Abcam), PDGF-C (Santa Cruz Biotechnology), and GFP (Medical & Biological Laboratories, Nagoya, Japan).

Statistical analysis

Data were expressed as mean ± s.e., and analyzed using GraphPad Prizm (GraphPad Software, San Diego, CA). The statistical significance was evaluated by one-way analysis of variance.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Table S1. Primer sequences used in quantitative PCR
Figure S1. Increased expression of platelet-derived growth factor (PDGF) isoforms and PDGF receptor (PDGFR) subunits following unilateral ureteral obstruction (UUO) surgery.
Figure S2. Coll-GFP transgene was a robust reporter for both *collagen 1* (*α1*) *transcript* and protein.
Figure S3. PDGF expression in kidneys.
Figure S4. Anti-PDGFR antibody specifically inhibited receptor activation in PDGF-stimulated kidney myofibroblasts.
Figure S5. Anti-PDGFR antibody decreased the expanded population of Coll-GFP+ cells in UUO kidneys day 4 after surgery.
Figure S6. Anti-PDGFR antibody attenuated kidney fibrosis.
Figure S7. Anti-PDGFR antibody inhibited injury-stimulated proliferation of pericytes/fibroblasts.
Figure S8. PDGFRα and PDGFRβ were not detected in bone marrow-derived macrophages and UUO kidney macrophages.
Figure S9. Imatinib inhibited population expansion of myofibroblasts in UUO kidneys.
Figure S10. Imatinib inhibited proliferation of pericytes/myofibroblasts in UUO kidneys.
Figure S11. Imatinib inhibited macrophage infiltration in UUO kidneys.
Figure S12. Imatinib attenuated obstructive kidney fibrosis.
Figure S13. C-kit did not express in pericytes and myofibroblasts.
Figure S14. Imatinib inhibited population expansion of interstitial Coll-GFP+ cells and attenuated kidney fibrosis induced by unilateral ischemia-reperfusion injury (IRI).
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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